

Delivery of SAR 1118 to the Retina via Ophthalmic Drops and its Effectiveness in a Rat Streptozotocin (STZ) Model of Diabetic Retinopathy (DR)

Vidhya R. Rao,¹ Elizabeth Prescott,² Namdev B. Shelke,¹ Ruchit Trivedi,¹ Peter Thomas,² Craig Struble,² Tom Gadek,^{*,3} Charles A. O'Neill,³ and Uday B. Kompella^{*,1}

PURPOSE. To determine the pharmacokinetics of SAR 1118, a small-molecule antagonist of leukocyte function-associated antigen (LFA)-1, after administration of ophthalmic drops in normal rats, and to determine its pharmacologic activity by assessing the inhibition of retinal leukostasis and vascular leakiness in a streptozotocin (STZ)-induced diabetic retinopathy model.

METHODS. The ocular pharmacokinetics of SAR 1118 were studied in rats after a single topical dose of ¹⁴C-SAR 1118 (1 mg/eye; 40 μ Ci; 15.5 μ L). SAR 1118 concentration time profiles in plasma and ocular tissues were quantified by liquid scintillation counting (LSC). The pharmacologic activity of SAR 1118 eye drops administered thrice daily for 2 months at 1% (0.3 mg/eye/d) and 5% (1.5 mg/eye/d) was assessed in an STZ-induced diabetic rat model by determining retinal leukostasis and blood-retinal barrier breakdown. Diabetic rats treated with periocularly administered celecoxib microparticles served as the positive control, and vehicle-treated rats served as the negative control.

RESULTS. A single dose of 6.5% ¹⁴C-radiolabeled SAR 1118 ophthalmic drops delivered retinal drug levels greater than 1 μ M in less than 30 minutes and sustained levels greater than 100 nM for 8 hours. SAR 1118 eye drops significantly reduced leukostasis and blood-retinal barrier breakdown in a dose-dependent manner.

CONCLUSIONS. SAR 1118 ophthalmic drops administered thrice daily deliver therapeutic levels of SAR 1118 in the retina and can alleviate the retinal complications associated with diabetes. (*Invest Ophthalmol Vis Sci.* 2010;51:5198–5204) DOI: 10.1167/iops.09-5144

Diabetic retinopathy (DR), a microvascular complication of diabetes, is a significant cause of acquired blindness worldwide.^{1,2} Clinical manifestations of DR include a progression to proliferative diabetic retinopathy (PDR), followed by

diabetic macular edema (DME), resulting in a moderate to severe vision loss as a result of pathologic angiogenesis of retinal blood vessels or leakage, respectively.³ Current therapies, including laser photocoagulation, anti-vascular endothelial growth factor molecules, and corticosteroids are beneficial in delaying the progression of the disease. However, their use is limited by severe side effects, as a result of either the invasive procedure or adverse ocular effects, including recurrent DME, glaucoma, or cataracts.^{2,4} Alternative or adjunct noninvasive pharmacotherapy is therefore of significant importance in the future management of DR.

DR is in part recognized as a chronic inflammatory disease. Various inflammatory processes including, upregulation of inflammatory mediators, infiltration of inflammatory cells, and retinal leukostasis have been observed in early stages of DR in humans and in diabetic animal models.^{5–7} Retinal leukostasis is a key step in the early inflammatory process and has been implicated in a cascade of retinal vascular events, including vascular endothelial cell death, thrombosis, nonperfusion, and ischemia, eventually leading to enhanced permeability of the blood-retinal barrier.⁸ Leukostasis involves leukocyte-endothelial cell adhesive interactions mediated by leukocyte function-associated antigen (LFA)-1 (CD11a/CD18), a cell surface adhesion molecule of the β_2 (CD18) family of integrin receptors expressed on leukocytes, and intercellular adhesion molecule (ICAM)-1, expressed by endothelial cells.⁹ Specific inhibition of leukocyte-endothelial cell interaction is therefore a potential target for reducing retinal leukostasis, subsequent blood-retinal barrier leakage, and edema.^{10,11}

SAR 1118 is a novel small-molecule antagonist of LFA-1. It is a member of the class of small-molecule LFA-1 antagonists shown to bind the I-domain of the CD11a subunit of LFA-1 and serves as a direct competitive antagonist of LFA-1 binding to ICAM-1.¹² The molecule potentially inhibits the binding of LFA-1 on Jurkat cells to ICAM-1 coated on a microtiter plate, with an IC₅₀ of 2 nM, and is capable of preventing leukocyte adhesion to endothelial cells in vivo (Murphy CJ, McIntyre K, Leatherberry G, et al., unpublished data, 2010). SAR 1118 has extraordinary solubility in excess of 100 mM in a saline-based ophthalmic drop formulation. The objective of the present study was twofold: to determine whether SAR 1118 can attain therapeutic levels (>2 nM) in the back of the eye when delivered as ophthalmic drops formulated at up to 100 mM and to assess whether repeated topical administration of SAR 1118 reduces retinal leukostasis and vascular leakage in a streptozotocin (STZ)-induced diabetic rat model of DR.

METHODS

Animals

Male Brown Norway (BN) rats weighing 175 to 225 g (Charles River Laboratories, Wilmington, MA) and male Sprague-Dawley (SD) rats

From the ¹Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, Colorado; ²Covance Laboratories Inc., Madison, Wisconsin; and ³SARcode Corporation, San Francisco, California.

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*Each of the following is a corresponding author: Uday B. Kompella, Department of Pharmaceutical Sciences, University of Colorado, 12700 East 19th Avenue, C238-P15, Aurora, CO 80045; uday.kompella@ucdenver.edu.

Tom Gadek, SARcode Corporation, 343 Sansome Street, Suite 505, San Francisco, CA 94104; gadek@sarcode.com.

weighing 231 to 294 g (Harlan Laboratories, Indianapolis, IN) were used in the experiments. The animals were maintained in a 12-hour light/12-hour dark cycle, with food and water provided ad libitum. The pharmacologic activity studies were conducted at the University of Colorado, and the pharmacokinetics studies were conducted at Covance Laboratories, Inc. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and institutional animal care and use guidelines.

Pharmacokinetics

The Sprague-Dawley rats received a single topical dose of radiolabeled [14 C]-SAR 1118 (Perkin Elmer Life Sciences, Waltham, MA) at a dose of 1 mg/eye (40 μ Ci/eye; dose volume, 15.5 μ L/eye; dose concentration, 100 mM, 6.5% wt/vol) in both eyes. Blood was collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hours after the dose and centrifuged to separate the plasma and cellular components. The animals were killed at 0.5, 2, 4, 8, 12, and 24 hours after the dose and the eyes collected. Drug levels in blood, plasma, cellular components, and ocular tissues, including the conjunctiva, cornea, lens, retina, choroid-RPE, sclera, aqueous humor, and vitreous humor were determined with a liquid scintillation counter (model 2900TR; Packard Instrument Co., Meriden, CT).

Experimental Diabetes Induction and Dose Administration

Diabetes was induced in a group of BN rats with a single intraperitoneal injection of 60 mg/kg STZ (cat. no. S0130; Sigma-Aldrich, St. Louis, MO) in 10 mM citrate buffer (pH 4.5), after an overnight fast. Twenty-four hours after STZ injection, the blood glucose levels in the animals were determined with a glucose monitor (One Touch; Life Scan Inc., Milpitas, CA). Animals with blood glucose levels greater than 250 mg/dL were considered diabetic. The animals were divided into groups 1 to 5. Group 1 comprised normal animals that were not diabetic. Group 2 comprised animals without treatment. Groups 3, 4, 5, and 6 included diabetic animals that were treated with vehicle, 1% SAR 1118, 5% SAR 1118, and celecoxib-poly(lactide-co-glycolide) microparticles, respectively. SAR 1118 at 1% or 5% wt/vol or vehicle was applied in topical drops to the right eye at a volume of 10 μ L/eye thrice daily (0.3 and 1.5 mg drug/eye/d in groups 4 and 5, respectively) with 6 to 8 hours between doses. A single dose of celecoxib microparticles was prepared (750 μ g of celecoxib; cat. no. C-1502; LC laboratories, Woburn, MA) and injected in the posterior subconjunctival space of the right eye of anesthetized rats, as described previously.¹³ After 2 months of treatment, the animals were killed and subjected to various assays to assess retinal leukostasis and blood-retinal barrier leakage.

Retinal Leukostasis

Retinal Leukostasis Assay. This assay was performed as described previously, with some modifications.¹⁴ The animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). The chest cavity was carefully opened, and the animals were perfused via the left ventricle with phosphate-buffered saline (PBS, pH 7.4; 250 mL/kg body weight). The animals were then perfused with FITC-conjugated concanavalin A lectin (40 μ g/mL in PBS [pH 7.4], 5 mg/kg; cat no. FL1001; Vector Laboratories, Burlingame, CA) to label the adherent leukocytes and the vascular endothelial cells. The residual unbound lectin was removed by PBS perfusion. The eyes were collected and fixed in 2% paraformaldehyde, and the retinas were carefully removed and flat mounted. Leukocyte adherence to vessel walls was monitored with a fluorescence microscope (Digital Eclipse C1; Nikon Inc., Melville, NY). The total number of leukocytes adhering within the entire retinal vasculature was counted and compared between the normal, nondiabetic, and diabetic groups treated with vehicle, SAR 1118 1% wt/vol, SAR 1118 5% wt/vol, and celecoxib microparticles.

Myeloperoxidase (MPO) Assay. MPO activities in the retinas were determined with an MPO fluorometric detection kit (Assay Designs, Ann Arbor, MI). Briefly, the retinas were isolated from the eyes, homogenized, and sonicated in 250 μ L of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. Sonicated homogenates were subjected to three freeze-thaw cycles. The samples were centrifuged at 15,000g at 4°C for 15 minutes. The MPO activity in the supernatant was estimated by using a standard curve generated as instructed by the manufacturer.

Blood-Retinal Barrier Leakage

FITC-Dextran Leakage Assay. Blood-retinal barrier leakage was assessed by FITC-dextran leakage assay that was a modification of a published method.¹⁵ Briefly, the animals were anesthetized as previously described. FITC-dextran (4.4 kDa, 50 mg/mL in PBS, 50 mg/kg body weight; cat. no. FD4; Sigma-Aldrich, St. Louis, MO) was injected intravenously. After 10 minutes (circulation time for FITC-dextran), the blood samples (~1 mL) were collected in EDTA containing centrifuge tubes. The chest cavity was opened, and the animals were perfused with PBS (500 mL/kg body weight). The eyes were collected and the retinas were isolated and homogenized in 1 mL of PBS. The homogenates were clarified by centrifugation at 15,000g, 4°C for 15 minutes. The relative FITC fluorescence units in the supernatant were measured by using a spectrofluorometer (SpectraMax; Molecular Devices; Sunnyvale, CA) at an excitation wavelength of 483 nm and an emission wavelength of 538 nm. The amount of FITC-dextran in the retina samples was estimated from the standard curve generated from known amounts of FITC-dextran and normalized to the retinal weight and the plasma concentration of the FITC-dextran. The blood-retinal barrier leakage was calculated using the formula:

$$\frac{\text{Retinal FITC} - \text{dextran } (\mu\text{g})/\text{retinal weight (g)}}{\text{Plasma FITC} - \text{dextran } (\mu\text{g}/\mu\text{L}) \times \text{circulation time (min)}}$$

Vitreous-Plasma Protein Ratio. The vitreous-to-plasma protein ratio was determined as described previously.¹³ The blood samples (1 mL) were collected in EDTA-containing tubes and centrifuged at 15,000g for 15 minutes at 4°C to separate the supernatant plasma. The frozen vitreous from each eye was isolated in tubes (Eppendorf, Fremont, CA) and thawed at room temperature to liquefy. The vitreous samples were centrifuged at 15,000g for 15 minutes at 4°C. The protein in the vitreous samples and plasma was measured with a Bradford kit (Fermentas Life Sciences, Glen Burnie, MD).

Data Analysis

Data are expressed as the mean \pm SD. The comparisons between the six groups were made by one-way ANOVA followed by the Tukey post hoc analysis. The results were considered statistically significant at $P < 0.05$.

RESULTS

Pharmacokinetics Study

After topical ocular administration of [14 C]-SAR 1118, the maximum postdose concentrations were observed at 0.5 hour in all ocular tissues (Fig. 1). The average postdose radioactivity (nanogram equivalents [14 C]-SAR 1118/g) at 0.5 hour were in the following order: bulbar conjunctiva (31,500) > palpebral conjunctiva (26,300) > cornea (17,150) ~ iris ciliary body (ICB, 17,550) > sclera (2,750) > aqueous humor (1,770) > vitreous humor (1,330) > retina-choroid/RPE (510) > lens (38.5). Radioactivity was detected at all time points in the bulbar conjunctiva, palpebral conjunctiva, cornea, aqueous humor, vitreous humor and sclera with an average concentration of 1014, 423, 207, 19.9, 37.3 and 229 ng equivalents of [14 C]-SAR 1118/g at 24 hours after administration, respectively.

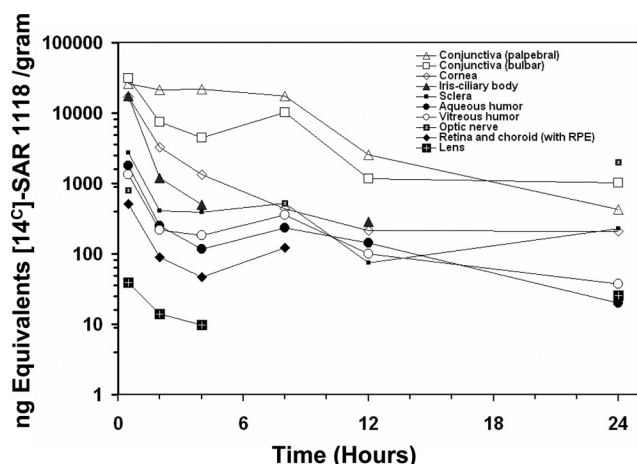


FIGURE 1. Concentrations of radioactivity in ocular tissues at specified times after a single topical application of [^{14}C]-SAR 1118 in male SD rats. Both eyes of normal SD rats (200–300 g body weight) were given a single topical application of radiolabeled [^{14}C]-SAR 1118 at a dose of 1 mg/eye (40 μCi /eye, dose volume: 15.5 μL /eye). After 0.5, 2, 4, 8, 12, and 24 hours, the animals were killed, and the level radiolabeled drug was measured in various ocular tissues. The data represent the mean concentration of the drug in both eyes ($n = 2$ eyes) and are expressed as the nanogram equivalent of [^{14}C]-SAR 1118 in the ocular tissues at specific time points.

Radioactivity approached the limit of quantification for iris ciliary body at 12 hours after the dose. Lens levels reached below detection limit at 8 and 12 hours and was again detected at 24 hours with an average concentration of 25.8 ng equivalents of [^{14}C]-SAR 1118/g at 24 hours after administration. Optic nerve head levels reached the limit of quantification at all time points except for 0.5, 8, and 24 hours with an average concentration of 796, 523, and 1990 ng equivalents of [^{14}C]-SAR 1118/g, respectively. The retinal radioactivity was detected up to 8 hours after application, with an average of 122 ng equivalents of [^{14}C]-SAR 1118/g (Fig. 1). These results suggest that greater than 100 nM retinal concentrations of SAR 1118 could be achieved after a single topical administration from a 100-mM dose. The plasma concentration after ocular topical administration was maximal at 0.25 hour with 194-ng equivalents [^{14}C]-SAR 1118/g, suggesting that the drug exhibits very low systemic exposure after ocular administration (Fig. 2).

Retinal Leukostasis

Retinal Leukostasis Assay. Figure 3A shows a representative confocal image of the diabetic retina, demonstrating leukocytes adhering to the retinal vasculature. The total number of adherent leukocytes in the various groups is presented in Figure 3B. The normal retinas had a mean adherent leukocyte count of 35.53 ± 8.1 . Diabetic retinas showed a mean adherent leukocyte count of 104.6 ± 11.2 ($P < 0.001$), indicating a threefold increase in adherent leukocytes in diabetic retinas. The diabetic retinas treated with 1% wt/vol SAR 1118, 5% wt/vol SAR 1118, and celecoxib microparticles had mean adherent leukocyte counts of 78.5 ± 13.4 ($P = 0.024$), 47.5 ± 8.2 ($P < 0.001$), and 64.25 ± 13.74 ($P < 0.001$), indicating up to 24.9%, 54.56%, and 38.57% inhibition of leukostasis in diabetic retinas, respectively.

MPO Activity. MPO activity in various groups is presented in Figure 3C. The normal retinas demonstrated a mean MPO activity of 0.13 ± 0.02 U/g retinal tissue. Diabetic retinas demonstrated mean retinal MPO activity of 0.74 ± 0.07 U/g retinal tissue, indicating a greater than fivefold ($P < 0.001$) increase in MPO activity in diabetic retinas. The diabetic reti-

nas treated with 1% wt/vol SAR 1118, 5% wt/vol SAR 1118, and celecoxib microparticles had mean MPO activity of 0.66 ± 0.08 , 0.53 ± 0.09 ($P = 0.044$), and 0.42 ± 0.13 ($P = 0.027$) U/g retinal tissue, respectively, indicating up to 10.8%, 28.3%, and 43.24% inhibition in MPO activity in diabetic retinas treated with 1% wt/vol or 5% wt/vol SAR 1118 and celecoxib microparticles, respectively.

Blood–Retinal Barrier Leakage

FITC-Dextran Leakage Assay. The FITC dextran leakage expressed as $\mu\text{g/g}$ retina/min circulation time is presented in Figure 4A for the various treatment groups. The FITC-dextran leakage in the normal retinas was in the range of 2 to 6 $\mu\text{g/g}$ retina/min circulation time. Diabetic retinas showed a rate of FITC-dextran leakage in the range of 90 to 100 $\mu\text{g/g/min}$ ($P < 0.001$). Diabetic retinas treated with 1% wt/vol SAR 1118, 5% wt/vol SAR 1118, or celecoxib microparticles demonstrated a mean FITC-dextran leakage of 67.17 ± 27.6 , 42.07 ± 10.8 ($P = 0.017$) and 39.09 ± 23.8 $\mu\text{g/g/min}$, indicating up to 25.36%, 53.25%, and 56.56% inhibition of FITC-dextran leakage, respectively. Retinas isolated from eyes treated with vehicle or contralateral retinas not treated with the test articles demonstrated FITC-dextran leakage similar to diabetic retinas.

Vitreous–Plasma Protein Ratio. The vitreous–plasma protein ratio for various treatment groups is presented in Figure 4B. Normal retinas had a mean vitreous–plasma protein ratio of 0.12 ± 0.04 . Diabetic retinas had a mean vitreous–plasma protein ratio of 0.47 ± 0.1 indicating about a fourfold increase in blood–retinal barrier leakage in diabetic retinas ($P < 0.001$). The diabetic retinas treated with 1% or 5% wt/vol SAR 1118, celecoxib microparticles had a mean vitreous plasma protein ratio of 0.39 ± 0.03 , 0.32 ± 0.07 , and 0.14 ± 0.05 ($P < 0.001$), suggesting up to 17.02%, 31.91%, and 70.21% inhibition of blood–retinal barrier leakage, respectively. Contralateral eyes not treated with the test articles or eyes treated with vehicle demonstrated vitreous–plasma protein ratios sim-

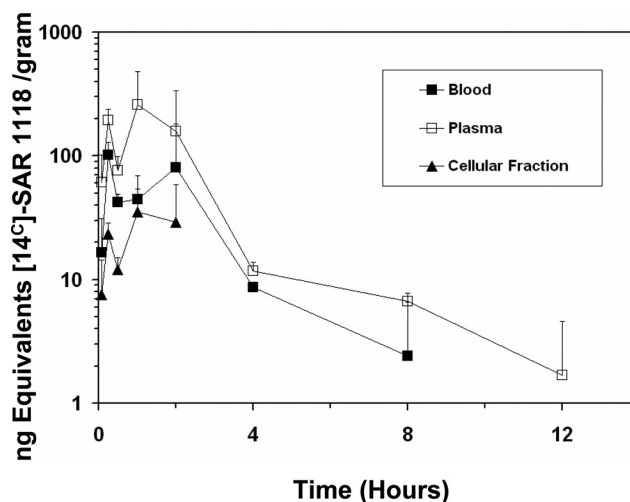
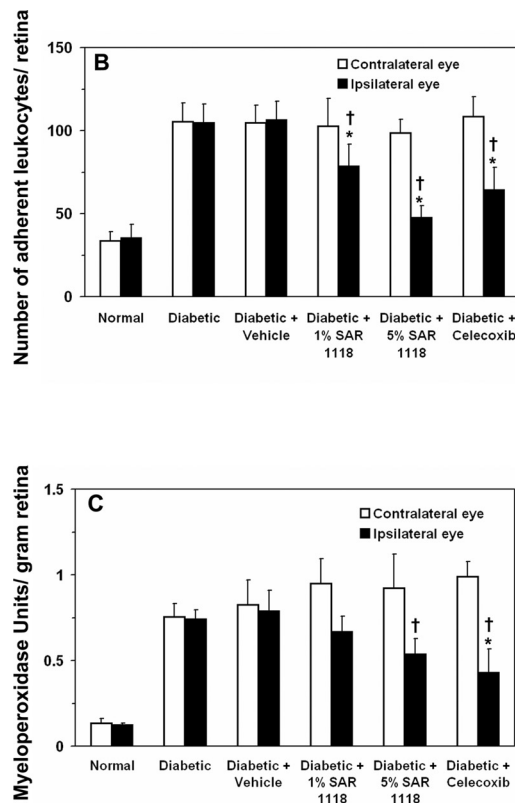
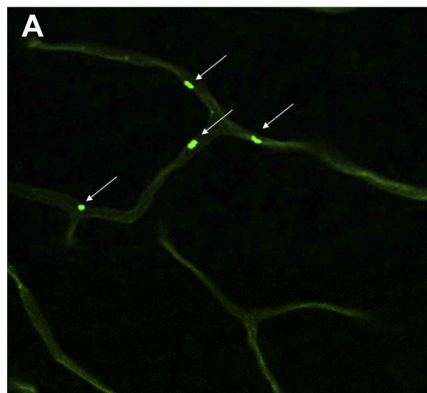


FIGURE 2. Concentrations of radioactivity in blood, plasma, and blood cellular fraction at specified times after a single topical application of [^{14}C]-SAR 1118 in male SD rats. Both eyes of normal SD rats (200–300 g body weight) were given a single topical application of radiolabeled [^{14}C]-SAR 1118 at a dose of 1 mg/eye (40 μCi /eye, dose volume: 15.5 μL /eye). After 0.083, 0.5, 2, 4, 8, 12, 24, and 72 hours, the level of the radiolabeled drug was measured in plasma, blood, and blood cells. The drug declined below the level of quantification beyond 12 hours after administration. The data represent the mean concentration of the drug ($n = 3$ rats; mean \pm SD) and are expressed as the nanogram equivalent of [^{14}C]-SAR 1118 at specific time points.

FIGURE 3. Topical SAR 1118 reduced retinal leukostasis in the retinal vasculature of diabetic rats. **(A)** Representative images of adherent leukocytes in diabetic retinal vasculature as observed by confocal microscope. *Arrows*: adherent leukocytes. Magnification, $\times 20$. **(B)** Total number of adherent leukocytes in the entire retina, isolated from the ipsilateral and contralateral eyes of normal ($n = 4$), diabetic ($n = 5$), diabetic+vehicle ($n = 6$), diabetic+1% SAR1118 ($n = 6$), diabetic+5% SAR 1118 ($n = 6$), and diabetic+celexib microparticle ($n = 4$) treatment groups. **(C)** Retinal MPO activity in retinas isolated from the ipsilateral and contralateral eyes of normal ($n = 6$), diabetic ($n = 6$), diabetic+vehicle ($n = 6$), diabetic+1% SAR1118 ($n = 6$), diabetic+5% SAR 1118 ($n = 6$), and diabetic+celexib microparticle ($n = 4$) treatment groups. All treatments were applied to the ipsilateral eyes of diabetic animals, and the parameters were tested after 2 months of treatment. Data are expressed as the mean \pm SD. *Significantly different from the diabetic group. †Significantly different from the vehicle-treated group.



ilar to diabetic retinas. Further, the plasma protein levels did not differ between the normal and diabetic animals.

DISCUSSION

In the present study, we demonstrated that SAR 1118, a novel small-molecule antagonist of LFA-1, is capable of achieving therapeutic concentrations in the retina after administration as a topical drop. This study is the first to demonstrate that a novel small-molecule antagonist specifically designed to inhibit leukocyte adhesion reduces diabetes-associated retinal leukostasis and blood-retinal barrier leakage after ophthalmic drop administration. Thus, we have linked the ocular pharmacokinetics of SAR 1118 to its pharmacodynamic effectiveness in the rat STZ model of DME.

Ophthalmic drops are noninvasive and the most desirable route for treating ocular diseases. However, the use of drops for treating retinal diseases such as age-related macular degeneration and DME has been limited by inefficient delivery of drugs to the retina.¹⁵ Several preclinical studies have demonstrated that topical ocular drops of various drug molecules, including small molecules such as nepafenac^{14,16} and large peptides such as insulin,^{17,18} can accumulate at effective concentrations in the retina and exert pharmacologic effects. It has been suggested that topically administered drugs can reach the retina via the conjunctiva, followed by transit across the sclera and underlying barriers.¹⁵ The molecule under investigation, SAR 1118, exhibits aqueous solubility greater than 100 mg/mL, facilitating its formulation as a concentrated solution of >100 mM that can be applied topically. A drug with high solubility has significant concentration gradients and hence greater rates of drug delivery across barriers. A study of the pharmacokinetics of the molecule demonstrated that, after a single topical application of 1 mg SAR 1118, greater than 1- μ M concentrations could be achieved within 30 minutes in the retinal tissue. The retinal levels were also sustained at concentrations greater

than 100 nM for 8 hours after a single topical administration. The peak plasma concentration (194-ng equivalents [¹⁴C]-SAR1118/g) of SAR 1118 after topical administration was >100 times lower than the peak conjunctival concentration (35,000 ng/g), suggesting that systemic exposure to the drug after topical administration is very low. Given the high levels of the drug (> 5000 ng equivalents [¹⁴C]-SAR1118/g) sustained for more than 8 hours in the bulbar conjunctiva and the high aqueous solubility of the drug, transscleral mechanisms could contribute to sustaining retinal levels of SAR 1118 above 100 nM. These levels are significantly above the reported IC₅₀ of 2 nM (Murphy CJ, McIntyre K, Leatherberry G, et al., unpublished data, 2010 and Ref. 12) for the *in vitro* inhibition of Jurkat T-cell attachment to ICAM-1, and thus SAR 1118 may be expected to demonstrate reduced leukostasis in the retina. This finding led us to adopt a thrice-daily dose regimen for our *in vivo* pharmacologic assessment studies in a diabetic rat model.

Retinal leukostasis, characterized as adhesion and entrapment of leukocytes in the retinal vasculature, is a key inflammatory process and an underlying cause of diabetic retinal complications, including, retinal vessel occlusion, nonperfusion, retinal endothelial cell death, and blood-retinal barrier leakage.^{19–22} In the STZ-induced diabetic rat model, leukostasis has been observed as early as 3 days after diabetes induction and is spatially and temporally associated with capillary nonperfusion and blood-retinal barrier breakdown.^{11,23} After topical administration of SAR 1118, we observed a significant decrease in the number of adherent leukocytes, visualized by FITC-concanavalin A binding in the retinal vasculature in comparison to that in diabetic nontreated retinas. Up to a 24.9% and 54.58% decrease in retinal leukostasis was observed in rats treated with 1% and 5% SAR 1118, respectively, establishing a dose-dependent decrease in retinal leukostasis. Furthermore, 5% SAR 1118 was able to reduce retinal leukostasis in diabetic retinas (47.5 ± 7.41) to levels comparable to that in the normal

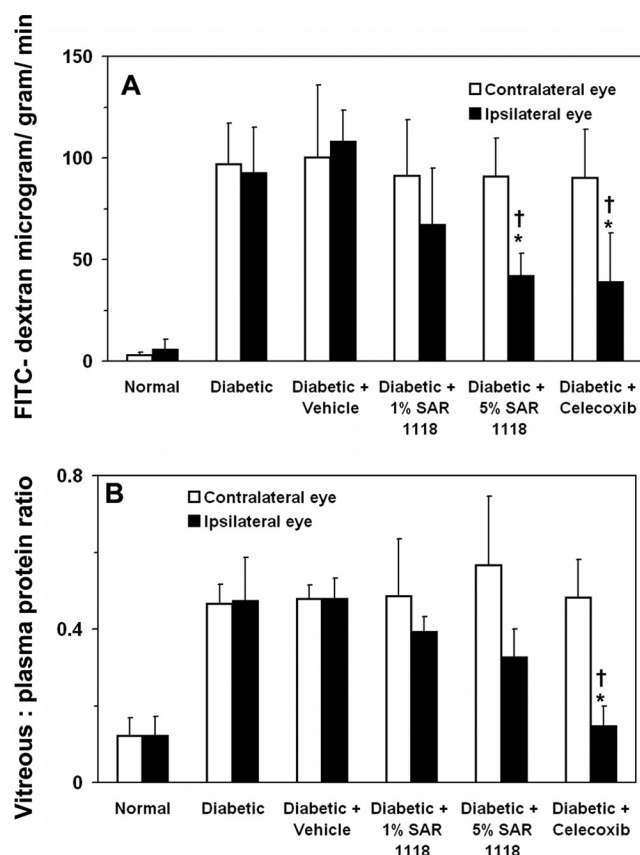


FIGURE 4. Topical SAR 1118 reduced the blood-retinal barrier breakdown in diabetic rats. (A) Retinal FITC-dextran leakage and (B) vitreous-plasma protein ratio of ipsilateral eye and the contralateral eye of normal ($n = 6$), diabetic ($n = 6$), diabetic+vehicle ($n = 6$), diabetic+1% SAR1118 ($n = 6$), diabetic+5% SAR 1118 ($n = 6$), and diabetic+celecoxib microparticle ($n = 4$) groups. Data are expressed as the mean \pm SD. All treatments were applied to the ipsilateral eye of diabetic animals, and the parameters were tested after 2 months of treatment. *Significantly different from diabetic group. †Significantly different from the vehicle-treated group.

retinas (35.5 ± 8.18). The interaction of the endothelial cell surface adhesion molecule ICAM, with the leukocyte integrins, in particular with the $\beta 2$ (CD18) subunit of the integrin molecule, expressed on various leukocytes, is important for the adhesion of leukocytes to endothelial cells.⁹ Enhanced expression of both endothelial ICAM and leukocyte CD18 is observed in DR.^{9,11,24} A recent study correlated the vitreous levels of ICAM to DME severity.²⁵ A previous study reported that mice lacking CD18 and ICAM have significantly fewer adherent leukocytes in retinal vasculature after diabetes induction with STZ.⁷ Also, diabetic animals treated with an intraperitoneal injection of anti-ICAM-1 mAb demonstrated a decrease in leukostasis by 48.5% and blood-retinal barrier leakage by 85.6%.¹¹ A significant decrease in leukostasis by 62% was also observed in an in vivo model of experimentally induced diabetes, after an intravenous injection of anti-CD18 F(ab9)2 fragments.⁹ In the present study, after topical administration of 5% SAR 1118 thrice daily, we observed a significant reduction in leukostasis by 54.58% ($P < 0.001$), similar to levels of inhibition seen with the IP injection of anti-ICAM mAb. The present study is the first to describe a molecule designed to mimic the LFA-1 binding epitope of ICAM-1 within a small-molecule framework,¹² which can selectively reduce retinal leukostasis in diabetic retinas to a level comparable to that in normal retinas, after an ocular topical drop administration. MPO is a heme protein

expressed in polymorphonuclear leukocytes (PMNs), including neutrophils, monocytes, and activated macrophages. Increases in leukostasis, leukocyte infiltration, and leukocyte-derived MPO have been implicated in the vascular dysfunction associated with diabetes and atherosclerosis.^{26–29} An increase in tissue MPO activity is representative of an increase in tissue PMNs and has been used for measuring leukocyte infiltration and adhesion.³⁰ Previously, enhanced in vitro neutrophil adhesion to retinal endothelial cells was demonstrated by measuring MPO activity in response to elevated glucose and insulin.^{31,32} In the present study, diabetic retinas demonstrated a greater than fourfold increase in retinal MPO activity compared with the normal nondiabetic retinas. Treatment with 1% and 5% SAR 1118 reduced the MPO activity by 10.8% and 28.3%, respectively. The reduction in MPO activity after SAR 1118, however, was not as statistically significant ($P < 0.05$) as the leukostasis inhibition ($P < 0.001$). This result may be attributable to the fact that LFA-1 antagonists are known to be more effective in inhibiting lymphocyte adhesion relative to PMNs (e.g., neutrophils),³³ and thus the higher residual MPO activity may reflect the preferential retinal infiltration of PMNs.^{31,32}

Blood-retinal barrier breakdown and the resulting diabetic macular edema is the major cause of vision loss in DR. Inhibition of leukostasis reduces blood-retinal barrier breakdown in animal models of DR.^{11,13,19} Extravasation of systemically perfused FITC-dextran (4.4 kDa) into retinal tissues and an increase in the vitreous-plasma protein ratio are indicative of blood-retinal barrier dysfunction and enhanced blood-retinal barrier breakdown as a result of diabetes. In the present study, diabetic retinas without any treatment demonstrated an 8- to 10-fold increase in FITC-dextran leakage in comparison to normal retinas. A decrease in FITC-dextran leakage by 25.36% and 53.25% was observed after treatments with 1% and 5% SAR 1118, respectively, suggesting a dose-dependent decrease in blood-retinal barrier breakdown after SAR treatment. The increase in the vitreous versus plasma protein ratio in diabetic rats is also consistent with blood-retinal barrier breakdown.^{33,34} Diabetic eyes had an approximately fourfold increase in vitreous plasma protein ratios and a dose-dependent decrease of 17% and 32% was observed in this ratio after treatment with 1% and 5% SAR 1118, respectively.

The present study is the first to demonstrate that SAR 1118, an agent designed to selectively prevent leukocyte adhesion via ICAM, can inhibit retinal leukostasis and subsequent vascular leakage after topical administration. SAR 1118 may exert its beneficial effects in diabetic retinas through direct inhibition of leukostasis as well as inhibition of cytokine and inflammatory mediator production. In several studies, Joussen et al.^{7,35,36} reported that, in addition to VEGF expression, ICAM-1 expression as well as TNF α can contribute to the complications of DR. For instance, ICAM-1-knockout diabetic mice were less susceptible to diabetes-associated retinal complications, including enhanced retinal leukostasis and blood-retinal barrier leakage.⁷ Further, a thrice-weekly intraperitoneal injection of a monoclonal antibody against ICAM-1 reduced leukostasis by 40% and blood-retinal barrier breakdown by 83% at the end of 1 week in diabetic animals.¹¹ Thus, it is through its ability to inhibit the interaction of ICAM-1 and LFA-1 that SAR 1118 is thought to exert its beneficial effects in DR. Also, a soluble TNF α receptor/Fc receptor construct (etanercept) was shown to inhibit³⁵ retinal leukostasis and blood-retinal barrier leakage without affecting the retinal VEGF levels in STZ-induced diabetic rats. In addition, anti-inflammatory molecules inhibiting retinal prostaglandins are capable of reducing retinal leukostasis and blood-retinal barrier breakdown. A 0.5% topical drop formulation of nepafenac sodium, has been shown to reduce leukostasis and inflammatory markers after four times daily administration at

the end of 2 and 9 months, respectively.¹⁴ Since, SAR 1118 inhibits TNF α secretion from peripheral blood mononuclear cells stimulated with staphylococcal enterotoxin B with an EC₅₀ of 76 nM (Murphy CJ, McIntyre K, Leatherberry G, et al., unpublished data, 2010) inhibition of TNF α -mediated effects is an additional mechanism by which SAR 1118 may exert its beneficial effects in DR. Of interest, TNF α has been shown to upregulate VEGF receptor 2, thereby increasing the bioactivity of VEGF,³⁷ another key mediator of vascular disease underlying DR. Thus, SAR1118 may act by multiple mechanisms, including the inhibition of leukostasis, inhibition of TNF α mediated mechanisms as well as the interaction of TNF α and VEGF in alleviating diabetes-associated retinal complications.

Studies have shown that celecoxib, a nonsteroidal anti-inflammatory molecule and a selective cox-2 inhibitor, is beneficial in alleviating DR-associated blood-retinal barrier breakdown in a diabetic rat model.^{13,38} A single periocular injection of celecoxib microparticles sustained therapeutic levels of the drug in the eye and was effective in reducing the blood-retinal barrier breakdown, even at the end of 2 months.¹³ Inhibition of retinal leukostasis has also been observed after treatment with other NSAIDs including aspirin, nepafenac, and the selective cox-2 inhibitor meloxicam.^{7,14} We therefore used celecoxib, delivered by a single periocular injection of sustained-release microparticles, as a positive control for our experiments to determine the efficacy of SAR 1118 in a DR rat model. SAR 1118 after thrice-daily ocular topical application demonstrated comparable or greater efficacy in inhibiting retinal leukostasis. Thus, repeated topical drops can be as effective as the more invasive administration of a slow-release system. Alternatively, SAR 1118 can potentially be administered in microparticle formulation via an invasive mode for sustained drug delivery and efficacy.

The primary purpose of the pharmacokinetics study was to demonstrate that the drug from eye drops reaches tissues in the posterior eye. For a single-dose pharmacokinetics study, 6.5% SAR 1118—that is, a 100-mM concentration of SAR 1118 (mw 640, sodium salt)—was used (Fig. 1). Although the 5% dose strength used in the efficacy studies is slightly lower in concentration (78 mM vs. 100 mM) than the 6.5% solution used in the single-dose pharmacokinetics study, 5% SAR 1118 in efficacy studies was administered at 10 μ L thrice daily, with a daily dose of 1.5 mg/eye/d, which is a 1.5-fold higher drug exposure than that in the single-dose study. Thus, drug delivery to the back of the eye is expected to be of a similar order of magnitude in the efficacy study with 5% SAR 1118. The dose-dependent efficacy observed in the present study with 1% and 5% SAR 1118 suggests that there may be a dose-proportionate retinal delivery at various strengths of SAR 1118. In addition, during the efficacy studies, pharmacologic effects were local and limited to the treated eye. This result suggests that the SAR 1118 eye drops deliver the drug to the back of the eye largely via local pathways in achieving their pharmacologic effects.

In conclusion, we demonstrated that SAR 1118 a small anti-inflammatory molecule antagonist designed to inhibit the LFA-1-ICAM-1 interaction can be delivered at therapeutic levels to the retina after topical ocular administration and is effective in alleviating the DR associated retinal leukostasis and blood-retinal barrier breakdown in a diabetic rat model. The results suggest that SAR 1118, a small-molecule LFA-1 antagonist, may be beneficial in treating vision loss in diabetic patients.

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